

STARCH-EXCESS4 Is a Laforin-Like Phosphoglucan Phosphatase Required for Starch Degradation in *Arabidopsis thaliana*

Oliver Kötting,^{a,1,2} Diana Santelia,^{a,1} Christoph Edner,^b Simona Eicke,^a Tina Marthaler,^a Matthew S. Gentry,^c Sylviane Comparot-Moss,^d Jychian Chen,^e Alison M. Smith,^d Martin Steup,^b Gerhard Ritte,^{b,3} and Samuel C. Zeeman^a

^aInstitute of Plant Sciences, ETH Zurich, 8092 Zurich, Switzerland

^bPlant Physiology, Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam-Golm, Germany

^cDepartment of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky 40536-0509

^dDepartment of Metabolic Biology, John Innes Centre, Norwich NR4 7UH, United Kingdom

^eInstitute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan

Starch is the major storage carbohydrate in plants. It is comprised of glucans that form semicrystalline granules. Glucan phosphorylation is a prerequisite for normal starch breakdown, but phosphoglucan metabolism is not understood. A putative protein phosphatase encoded at the Starch Excess 4 (*SEX4*) locus of *Arabidopsis thaliana* was recently shown to be required for normal starch breakdown. Here, we show that *SEX4* is a phosphoglucan phosphatase in vivo and define its role within the starch degradation pathway. *SEX4* dephosphorylates both the starch granule surface and soluble phosphoglucans in vitro, and *sex4* null mutants accumulate phosphorylated intermediates of starch breakdown. These compounds are linear α -1,4-glucans esterified with one or two phosphate groups. They are released from starch granules by the glucan hydrolases α -amylase and isoamylase. In vitro experiments show that the rate of starch granule degradation is increased upon simultaneous phosphorylation and dephosphorylation of starch. We propose that glucan phosphorylating enzymes and phosphoglucan phosphatases work in synergy with glucan hydrolases to mediate efficient starch catabolism.

INTRODUCTION

Starch in plants and glycogen in animals represent the major storage carbohydrates of these organisms. In both polymers, glucose residues are linked by α -1,4-glycosidic bonds and branched via α -1,6-glycosidic linkages. Branching in amylopectin, the major component of starch, is nonrandom and results in clusters of chains at regular intervals along the axis of the molecule. Within the clusters, adjacent chains form double helices, which organize into lamellae. The alternation of these crystalline lamellae with amorphous lamellae containing the branch points accounts for the insoluble semicrystalline nature of starch granules (Smith et al., 2005; Zeeman et al., 2007). Glycogen has twice as many branch points as amylopectin and a branch point distribution that does not result in cluster formation. Consequently, glycogen does not form the same secondary structures as amylopectin and is soluble. Both glycogen and

starch also contain significant amounts of covalently linked phosphate, the significance of which has only recently become apparent (for review, see Blennow et al., 2002).

Transitory starch is a major product of photosynthetic carbon fixation of lower and higher plants. It accumulates inside leaf cell chloroplasts during the day and is hydrolyzed to maltose and glucose to support sucrose synthesis and sustain cell metabolism during the following night (Smith et al., 2005; Messerli et al., 2007). How starch degrading enzymes manage to act on the semicrystalline surface of a starch granule at night is not yet fully understood, but it seems likely that phosphorylation of amylopectin plays a crucial role in the initial attack at the granule (Lorberth et al., 1998; Blennow et al., 2002; Zeeman et al., 2004; Lloyd et al., 2005). Two starch phosphorylating enzymes are known in plants: glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD) (Lorberth et al., 1998; Yu et al., 2001; Ritte et al., 2002; Baunsgaard et al., 2005; Kötting et al., 2005). Both enzymes catalyze the transfer of the β -phosphate of ATP to a glucosyl residue of amylopectin (Ritte et al., 2002; Baunsgaard et al., 2005; Kötting et al., 2005). GWD exclusively phosphorylates the C-6 positions of glucosyl residues, whereas PWD phosphorylates the C-3 positions (Ritte et al., 2006). Starch from *Arabidopsis thaliana* *gwd* null mutants is essentially phosphate-free, whereas *pwd* starch is only phosphorylated at C-6 positions (Ritte et al., 2006). Using crystalline maltodextrins, evidence has been presented that the phosphorylation of insoluble glucans favors their solubilization (Hejazi et al., 2008).


¹ These authors contributed equally to this work.

² Address correspondence to koliver@ethz.ch.

³ Current address: Metanomics GmbH, 10589 Berlin, Germany.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Oliver Kötting (koliver@ethz.ch).

 Online version contains Web-only data.

 Open Access articles can be viewed online without a subscription. www.plantcell.org/cgi/doi/10.1105/tpc.108.064360

Therefore, it is likely that the charged and hydrophilic phosphate groups disturb the tight packing of the semicrystalline lamellae at the starch granule surface by partially unwinding the amylopectin double helices, thereby rendering them accessible for degrading enzymes (Yu et al., 2001; Kötting et al., 2005; Edner et al., 2007; Hejazi et al., 2008). Glucan hydrolyzing enzymes, including exoamylases (β -amylase), endoamylases (α -amylase), and debranching enzymes (isoamylase and limit dextrinase), are involved in the subsequent degradation of starch (for review, see Zeeman et al., 2007). The net products of the pathway (i.e., maltose and glucose) are exported from the chloroplast for metabolism in the cytosol. However, the fate of phosphoglucans during starch degradation in the chloroplasts is not yet understood.

Recently, a chloroplastic glucan binding phosphatase was described that is required for normal starch degradation in *Arabidopsis* (Niittylä et al., 2006). The enzyme is encoded at the Starch Excess 4 (*SEX4*; At3g52180) locus (also designated as *PTPKIS1* and *DSP4*) (Fordham-Skelton et al., 2002; Kerk et al., 2006; Niittylä et al., 2006; Sokolov et al., 2006). Like *gwd* and *pwd* mutants, *Arabidopsis* mutants lacking the *SEX4* protein are impaired in starch degradation during the night (Zeeman et al., 1998), leading to a progressive accumulation of starch as the leaves age (Zeeman and ap Rees, 1999). The *SEX4* protein contains a dual-specificity phosphatase domain and a carbohydrate binding module. Members of the dual-specificity phosphatase family have been shown to act on substrates as diverse as phosphoinositides, mRNA, and phosphoproteins, including phosphorylated protein kinases involved in signaling pathways (Luan, 2003; Alonso et al., 2004; Pulido and Hooft van Huijsduijnen, 2008). It was proposed that *SEX4* might dephosphorylate such a kinase, which would in turn modulate the activity of starch degrading enzymes (Kerk et al., 2006; Niittylä et al., 2006).

Remarkably, the most closely related protein to *SEX4* outside the kingdom Plantae is laforin, a phosphatase required for normal glycogen metabolism in vertebrates. Recessive mutations in the laforin gene (*EPM2A*) are one of the molecular reasons for Lafora disease (#OMIM 254780), an autosomal recessive neurodegenerative disorder resulting in severe epilepsy and death (Lafora and Glück, 1911; Minassian et al., 1998; Serratosa et al., 1999). The tissues of Lafora disease patients contain Lafora bodies (LBs), consisting of an aberrant and insoluble form of glycogen (Lafora and Glück, 1911; Harriman et al., 1955; Serratosa et al., 1995). Interestingly, LB structure appears to be more similar to amylopectin than to glycogen (Yokoi et al., 1967, 1968; Sakai et al., 1970).

Laforin is widely believed to be a phosphoprotein phosphatase involved in the regulation of glycogen-related enzymes (Liu et al., 2008; Singh et al., 2008; Solaz-Fuster et al., 2008). However, two recent studies showed that laforin is able to dephosphorylate solubilized amylopectin or glycogen in vitro and proposed that laforin is in fact a glucan phosphatase (Worby et al., 2006; Gentry et al., 2007). This is consistent with early studies showing that LBs contain significant amounts of phosphate (Schnabel and Seitelberger, 1968; Sakai et al., 1970) and the recent demonstration that the glycogen-bound phosphate is increased by up to fourfold in laforin-deficient mice (Tagliabracci et al., 2007).

Gentry et al. (2007) suggested that *SEX4* and laforin are functionally equivalent. Our work now provides direct evidence that *SEX4* is a phosphoglucan phosphatase in vivo, and we propose a physiological role for *SEX4* in the starch degradation pathway.

RESULTS

Recombinant *SEX4* Can Dephosphorylate the Starch Granule Surface

We tested the phosphatase activity of a recombinant *SEX4* enzyme preparation using intact starch granules from *Arabidopsis* leaves as substrate that were prephosphorylated in vitro by recombinant GWD and [β - ^{33}P]ATP (see Methods). Phosphate incorporation into starch by GWD was 17.3 μmol per mol Glc equivalents (i.e., 1 phosphate per 58,000 glucosyl residues). Although much lower than the level of phosphate in wild-type *Arabidopsis* starch (~ 1 in 2000 glucosyl residues) (Yu et al., 2001), the phosphate of in vitro-phosphorylated starch is located only on the surface of the granule and may thus be close to physiological levels in this location. *SEX4* protein was able to release a radioactive product from the granules with a specific activity of up to $5.6 \pm 0.5 \text{ nmol mg}^{-1} \text{ protein min}^{-1}$ (mean \pm SE, $n = 3$; Figure 1A). The specific phosphatase activity increased linearly with the amount of starch granules added to the assay. The radioactive product released by *SEX4* was shown to be ^{33}P -orthophosphate by thin layer chromatography followed by autoradiography (Figure 1B). The catalytically inactive protein *SEX4* C/S, which has a single amino acid exchange in the active site (Gentry et al., 2007), could not release significant amounts of phosphate from the granule surface (Figure 1A). These results are consistent with initial results using solubilized amylopectin in vitro (Gentry et al., 2007).

To determine if *SEX4* contributes significantly to phosphate release from granules in vivo, we incubated ^{33}P -labeled granules with leaf extracts of *sex4* mutants and the wild type. The amount of ^{33}P released by *sex4* extracts was 61% lower than that by wild-type extracts (Figure 1C), indicating that *SEX4* represents the major starch phosphatase activity in *Arabidopsis* leaves. Again, the released radioactivity was shown to be ^{33}P -orthophosphate by thin layer chromatography (Figure 1B).

The *sex4* Mutant Contains Elevated Levels of Phospho-Oligosaccharides

We analyzed whether loss of *SEX4* resulted in an increased phosphate content of starch granules in vivo. In fact, the phosphate content of isolated *sex4* starch was 31% lower than that of wild-type starch (Table 1). Zeeman et al. (2002b) showed that leaf starch from *sex4* mutants has more amylose than wild-type starch, and it is known that phosphate is exclusively esterified to the glucosyl units of amylopectin (Blennow et al., 2002). Therefore, we measured the amylopectin content of the wild-type and *sex4* starch preparations used for the phosphate determinations. Indeed, the amylopectin content of *sex4* starch was lower (65%) than that of wild-type starch (92%; Table 1). Normalizing the data

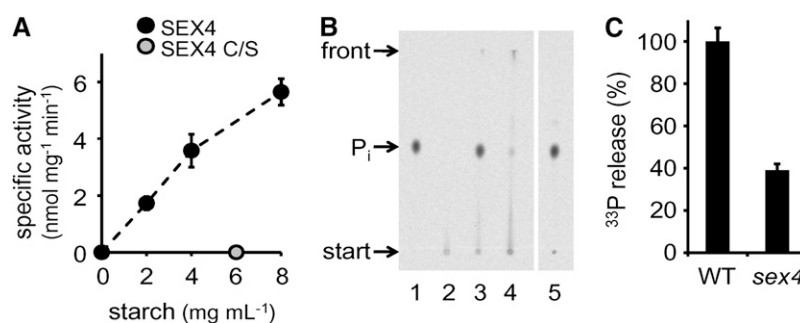


Figure 1. SEX4 Dephosphorylates the Starch Granule Surface.

(A) Specific activities of recombinant SEX4 (black circles; 0.5 $\mu\text{g mL}^{-1}$) and the active site mutant SEX4 C/S (gray circle; 0.5 $\mu\text{g mL}^{-1}$) incubated with different amounts of native starch granules that had been prephosphorylated in vitro by recombinant GWD and [β - ^{33}P]ATP as phosphate donor. Activity was measured as the release of ^{33}P from the granules into the supernatant. Values are means \pm SE; $n = 3$ independent experiments.

(B) Thin layer chromatography of the products released from ^{33}P -labeled starch granules by recombinant SEX4 protein (as in **[A]**) and leaf extracts of wild-type and *sex4* plants (as in **[C]**). Two microliters of each were analyzed as described (Hejazi et al., 2008). Arrows indicate start, front, and the position of orthophosphate. 1, recombinant SEX4; 2, medium control; 3, wild-type extract; 4, *sex4* extract; 5, hydrolyzed [β - ^{33}P]ATP. All samples were analyzed on the same plate.

(C) Quantification of released ^{33}P from in vitro prephosphorylated starch granules (as in **[A]**) by crude extracts from leaves of wild-type and *sex4* plants (0.22 mg total protein mL^{-1}). Values are means \pm SE; $n = 5$ independent experiments.

for equal amylopectin levels as shown in Table 1 revealed that amylopectin-bound phosphate is not significantly changed in *sex4* compared with the wild type.

Subsequently, we determined whether leaves from wild-type or *sex4* mutant plants contain different amounts or types of soluble phosphoglucans. To achieve this, analyses were performed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Our sample preparation for the HPAEC-PAD analysis of neutral oligosaccharides involves a purification step that would also remove phosphorylated compounds (see Methods). Therefore, we pretreated aliquots of the extracts with a phosphatase (Antarctic Phosphatase; New England Biolabs) prior to purification and HPAEC-PAD analysis. Untreated samples from both lines contained glucose and maltose and trace amounts of longer oligosaccharides. For wild-type plants, no additional peaks were detected in chromatograms of extracts after the phosphatase treatment (Figure 2A), indicating that phospho-oligosaccharides are either absent or below the detection limit. By contrast, following phosphatase treatment, the HPAEC-PAD chromatograms of *sex4* extracts contained many new peaks (Figure 2A). The same result was obtained when recombinant SEX4 protein was used instead of Antarctic Phosphatase (see Supplemental Figure 1 online). Incubation of phosphatase-treated *sex4* extracts with barley (*Hordeum vulgare*) β -amylase completely converted the compounds in the new peaks to maltose and maltotriose, indicating that they are linear malto-oligosaccharides (see Supplemental Figure 2 online). Comparison with external linear malto-oligosaccharide standards revealed that the degree of polymerization (DP) of the detected malto-oligosaccharides ranged from 4 to 20. However, the majority of malto-oligosaccharides had a DP of six or seven (Figure 2A).

To confirm the presence of phospho-oligosaccharides, we separated neutral and phosphorylated glucans from *sex4* leaf extracts on a preparative scale (see Methods). Subsequent mass

spectrometry (MS) analysis of the phosphorylated compounds revealed masses corresponding to phospho-oligosaccharides with DP 5 to 9, esterified with one or two phosphate groups (Figure 2B). To determine the position of the phosphates in the phospho-oligosaccharides, we incubated extracts of *sex4* leaves with barley β -amylase prior to phosphatase treatment and HPAEC-PAD analysis. No maltose release was detectable (see Supplemental Figure 2 online), indicating that the phosphate is located close to the nonreducing end of the phospho-oligosaccharides, since β -amylase releases maltose from the nonreducing end of maltodextrins but is blocked by phosphate esters in the vicinity of the target glucosidic bond (Takeda and Hizukuri, 1981). To quantify the phosphate present in phospho-oligosaccharides in *sex4* mutants, we measured the oligosaccharides detected by HPAEC-PAD by comparing them with external standards of glucose, maltose, and linear malto-oligosaccharides of DP 3 to 7, as described in Methods (Figure 2A). Based on the assumption that each oligosaccharide is singly phosphorylated, the total glucan-bound phosphate (i.e., the sum of the phosphate content of starch and phospho-oligosaccharides) was more than six times higher in *sex4* than in the wild type at the end of the day (Table 1). The amount of phosphate present in the phospho-oligosaccharide pool of *sex4* was more than three times larger than that in starch (Table 1). Given that we were able to detect doubly phosphorylated oligosaccharides (Figure 2B), these are likely to be slight underestimates of the total glucan-bound phosphate in *sex4*. Together, these data provide direct evidence that SEX4 is a phosphoglucan phosphatase in vivo.

Phospho-Oligosaccharides in *sex4* Are Produced during Starch Degradation and Accumulate as the Leaves Age

The phospho-oligosaccharide content of *sex4* leaves increased by 40% during the night when starch was broken down and decreased during the day when starch synthesis occurred

Table 1. Glucan-Bound Phosphate Content Is Higher in the *sex4* Mutant Than in Wild-Type Plants

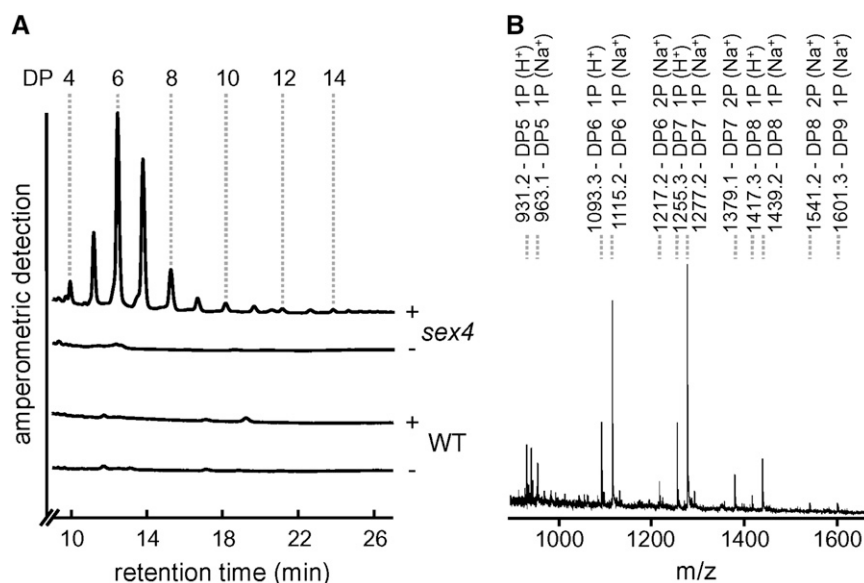
	Glucan-Bound Phosphate				
	(nmol P μmol^{-1} Glc Equivalents)		(nmol P g^{-1} FW)		
	Starch	Amylopectin	Starch	Phospho-Oligosaccharides	Total
Wild type	1.3 \pm 0.1	1.5 \pm 0.1	89.4 \pm 8.3	ND	89.4 \pm 8.3
<i>sex4</i>	0.9 \pm 0.0	1.4 \pm 0.1	134.7 \pm 5.6	452.2 \pm 82.5	586.9 \pm 109.8

Arabidopsis wild-type and *sex4* mutant plants were harvested at the end of the light period. Leaf starch was purified, and the amylopectin content was determined to be $91.8\% \pm 0.1\%$ and $65.2\% \pm 1.1\%$ for wild-type and *sex4* starch, respectively (mean \pm SE; $n = 3$). Starch-bound phosphate from the same starch preparation was determined. Quantification of starch and phospho-oligosaccharides on a fresh weight (FW) basis was done with a different batch of plants as described (see Methods; plants harvested at the end of the light period). Values represent the mean \pm SE ($n = 5$ individual plants). Note that the phosphate content of phospho-oligosaccharides is slightly underestimated because it was assumed that each phospho-oligosaccharide is only singly phosphorylated. ND, not detectable.

(Figure 3A). Since the starch excess phenotype of *sex4* mutants develops progressively as the leaves age (Zeeman and ap Rees, 1999), we investigated whether phospho-oligosaccharide levels also increase with leaf age (Figure 3B). Indeed, the phospho-oligosaccharide content was significantly higher in old *sex4* leaves compared with young leaves ($P = 0.0007$; Figure 3B). Neither the diurnal changes nor the changes with age were accompanied by significant changes in the phospho-oligosac-

charide chain length distribution. No phospho-oligosaccharides were detected in wild-type leaves.

Recently, it was shown that the starch excess phenotype of *sex4* plants could be complemented in stably transformed *sex4* plants expressing the SEX4 cDNA (Niittylä et al., 2006; Gentry et al., 2007). We determined the starch and phospho-oligosaccharide contents in two independent lines from one of the previous studies (Gentry et al., 2007). Expression of the SEX4

**Figure 2.** Phospho-Oligosaccharides Accumulate in Mutants Lacking SEX4.

(A) HPAEC-PAD analysis of wild-type and *sex4* extracts. Leaves were harvested at the end of the night and extracted in perchloric acid. Prior to purification by ion-exchange chromatography, neutralized extracts were treated with (+) or without (−) Antarctic Phosphatase and subsequently subjected to HPAEC-PAD analysis. One representative chromatogram each is shown ($n = 5$ individual plants). Numbers above the panel indicate the DP of the detected glucan chains. Essentially the same result was obtained when performing the phosphatase treatment with recombinant SEX4 instead of Antarctic Phosphatase (see Supplemental Figure 1 online).

(B) MS analysis of phospho-oligosaccharides enriched from *sex4* extracts. Whole rosettes of *sex4* plants were extracted in hot ethanol. Phospho-oligosaccharides were enriched on a Carbohydrate column and subjected to MS analysis. The mass-to-charge (m/z) values obtained reflect the theoretical masses of phospho-oligosaccharides with a DP of 5 to 9 glucose units, comprising one (1P) or two (2P) phosphate groups. Analytes were recovered as monosodium (Na^+) or monoprotonated (H^+) adduct ions. No phospho-oligosaccharides could be detected in wild-type extracts.

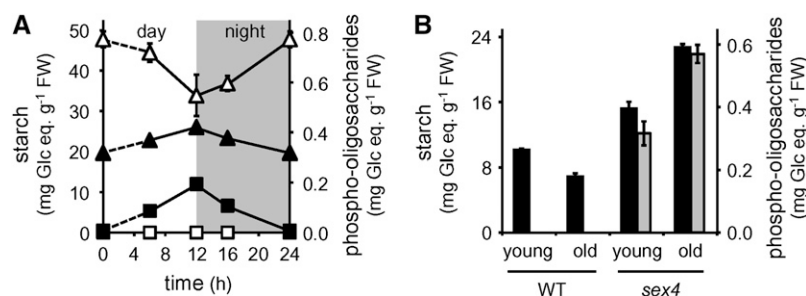


Figure 3. Phospho-Oligosaccharides in *sex4* Are Produced at Night and Accumulate during Development.

(A) Phospho-oligosaccharides (open symbols, right scale) and starch (closed symbols, left scale) levels of *sex4* (triangles) and wild-type (squares) plants were determined at different times during the diurnal cycle. Values are means \pm SE; $n = 5$ individual plants. Error bars not visible are smaller than the symbols. Values for 0 h are replotted from the 24 h time point and so do not represent actual values; indicated by the dashed lines. FW, fresh weight. **(B)** Whole rosettes comprising 18 to 24 leaves of wild-type and *sex4* plants were harvested at the end of the day and divided into two fractions. The first fraction comprised the eight youngest visible leaves pooled from two individual plants (young), and the second fraction contained the rest of the leaves from one plant (old). Starch (black bars; left scale) and phospho-oligosaccharide (gray bars; right scale) contents were measured. Values are means \pm SE; $n = 5$ biological replicates.

cDNA in *sex4* plants led to a partial complementation of the starch excess phenotype. The starch content at the end of the night decreased in both lines, by $84\% \pm 3\%$ and $65\% \pm 2\%$ (mean \pm SE; $n = 5$) compared with *sex4* plants (Figure 4). Phospho-oligosaccharides were reduced by $92\% \pm 2\%$ and $67\% \pm 4\%$ (mean \pm SE; $n = 5$) at the end of the day and $81\% \pm 4\%$ and $47\% \pm 13\%$ (mean \pm SE; $n = 5$) at the end of the night in these lines compared with *sex4* but showed the same trend in terms of increasing at night and decreasing during the day (Figure 4). The transformed line with the lower starch content also had the lower phospho-oligosaccharide content. Taken together, these data indicate that when *SEX4* is absent, phospho-oligosaccharides are produced during starch breakdown at night and that they accumulate progressively as the leaves age.

SEX4 Stimulates Starch Granule Degradation In Vitro

Edner et al. (2007) showed that hydrolysis of the granule surface in vitro by β -amylase 3 (BAM3) and isoamylase 3 (ISA3) is enhanced upon simultaneous starch phosphorylation by GWD. We investigated whether the addition of the *SEX4* phosphoglucan phosphatase to this system could further enhance granule hydrolysis in vitro. As reported previously, the total amount of glucan released from granules (including both phosphorylated and unphosphorylated glucans) increased twofold upon addition of GWD and ATP to a mixture containing phosphate-free starch granules, ISA3 and BAM3. Addition of *SEX4* protein to this mixture further doubled the rate of glucan release from the granule surface by BAM3 and ISA3 (Figure 5). Addition of *SEX4* protein had no effect in the absence of GWD, indicating that it is the phosphoglucan phosphatase activity of *SEX4* rather than the *SEX4* protein itself that results in increased granule degradation. We investigated whether the action of the hydrolytic enzymes affected the ability of *SEX4* to remove the ^{32}P added by recombinant GWD. Pretreatment of the labeled granules with BAM3 enhanced subsequent phosphate release from granules by *SEX4*

by more than fourfold (32.6 ± 1.1 compared with 6.9 ± 0.2 nmol mg^{-1} protein min^{-1} ; mean \pm SE, $n = 3$). These data indicate that in vivo glucan phosphorylation, hydrolysis, and dephosphorylation may be synergistic processes.

Mutation of *ISA3* or *AMY3* Enhances the Starch Excess Phenotype of *sex4* and Decreases the Accumulation of Phospho-Oligosaccharides

The accumulation of phospho-oligosaccharides in the *sex4* mutant shows that some glucan hydrolases can release glucans without the action of *SEX4*. In principal, two classes of enzymes are capable of releasing such phospho-oligosaccharides (i.e., debranching enzymes and endoamylases). The debranching enzyme *ISA3* was shown to be capable of releasing phospho-oligosaccharides from starch granules in vitro (Edner et al., 2007). To determine whether the phospho-oligosaccharides in *sex4* are produced by *ISA3*-mediated debranching, we selected the *sex4 isa3* double mutant. It contained significantly more starch than either of the single mutants (Figure 6), indicating that *ISA3* contributes to starch degradation in the *sex4* background. Furthermore, the phospho-oligosaccharide content was $63\% \pm 5\%$ and $67\% \pm 5\%$ lower (end of day and end of night, respectively; mean \pm SE, $n = 5$) in the *sex4 isa3* double mutant than in *sex4* (Figure 6), although the distribution of chain lengths was unchanged (Figure 7). The *isa3* single mutant did not contain detectable amounts of phospho-oligosaccharides. These data are consistent with the idea that *ISA3* liberates some, but not all, of the phospho-oligosaccharides present in *sex4* plants.

The only endoamylase shown to localize to chloroplasts in *Arabidopsis* is α -amylase 3 (*AMY3*) (Yu et al., 2005). To discover whether *AMY3*, like *ISA3*, is involved in the production of phospho-oligosaccharides in *sex4*, we selected the *sex4 amy3* double mutant. Loss of both *SEX4* and *AMY3* proteins resulted in a significantly higher starch content than in the *sex4* mutant (Figure 6). The total amount of phospho-oligosaccharides was

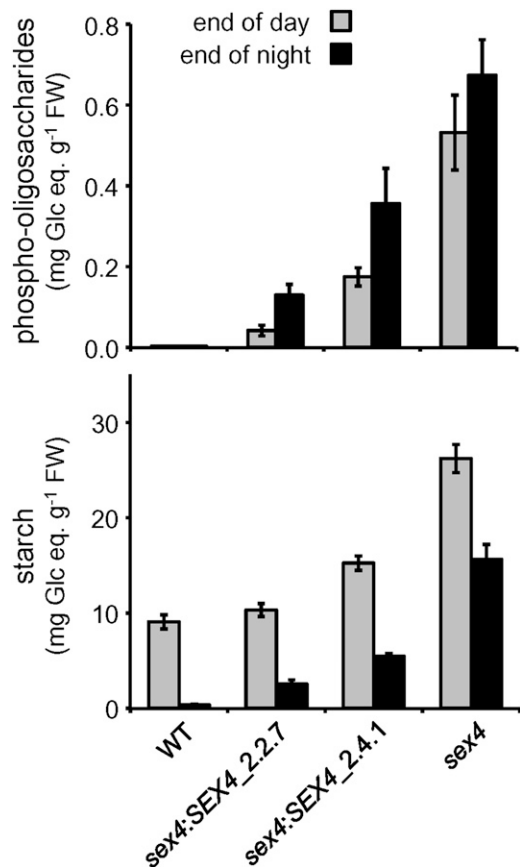


Figure 4. Expression of the SEX4 cDNA Partially Complements the *sex4* Phenotype.

Phospho-oligosaccharides and starch contents at the end of the day (gray bars) and the end of the night (black bars) were determined in wild-type and *sex4* plants as well as in two independent *sex4* lines expressing the *SEX4* cDNA under control of the cauliflower mosaic virus 35S promoter (*sex4:SEX4_2.2.7*, *sex4:SEX4_2.4.1*). Values represent the mean \pm SE of at least five biological replicates.

67% \pm 4% and 66% \pm 4% lower (end of day and end of night, respectively; mean \pm SE, n = 5) in *sex4 amy3* than in *sex4* plants (Figure 6). Additionally, more long phospho-oligosaccharides (DP 15 to 25) were detected in *sex4 amy3* plants than in *sex4* plants at both the end of the night (Figure 7) and the end of the day. These data suggest that AMY3 contributes to starch breakdown and the release of phospho-oligosaccharides in the *sex4* mutant background. However, starch levels were the same in the *amy3* single mutant and in wild-type plants (as observed previously) (Yu et al., 2005), and no phospho-oligosaccharides were detectable in this mutant (Figure 6).

To determine conclusively whether the phospho-oligosaccharides in the *sex4* mutant are derived from phosphorylated starch, we analyzed two additional double mutants. First, we crossed *sex4* to *pgm*, a line lacking chloroplastic phosphoglucomutase and consequently deficient in starch synthesis (Caspar et al., 1985). Like *pgm*, the *sex4 pgm* double mutant also contained

<1% of the starch of wild-type leaves, and phospho-oligosaccharides were not detectable (Figure 6). Second, we analyzed the *sex4 gwd* double mutant, which, as expected, contained very high levels of starch but no detectable phospho-oligosaccharides (Figure 6).

DISCUSSION

Taken together, our data provide compelling evidence that SEX4 is a phosphoglucan phosphatase that is required during starch degradation. We show that recombinant SEX4 releases phosphate directly from a physiological substrate—the granule surface that has been phosphorylated by GWD (Figure 1). Moreover, the markedly lower phosphate release by crude extracts of *sex4* leaves compared with wild-type extracts demonstrates that SEX4 represents the major granule phosphatase activity in leaves. The accumulation of phospho-oligosaccharides and the high levels of total glucan-bound phosphate in *sex4* mutants show that SEX4 is a phosphoglucan phosphatase in vivo. Our genetic and biochemical experiments show that the phospho-oligosaccharides are starch degradation products. First, phospho-oligosaccharides do not accumulate in *sex4 pgm* double mutants (Figure 6), showing that starch production is a prerequisite for the phospho-oligosaccharide accumulation. Second, the *sex4 gwd* double mutant also lacks phospho-oligosaccharides (Figure 6), indicating that GWD is required for generating the phosphoglucans from which the phospho-oligosaccharides are derived.

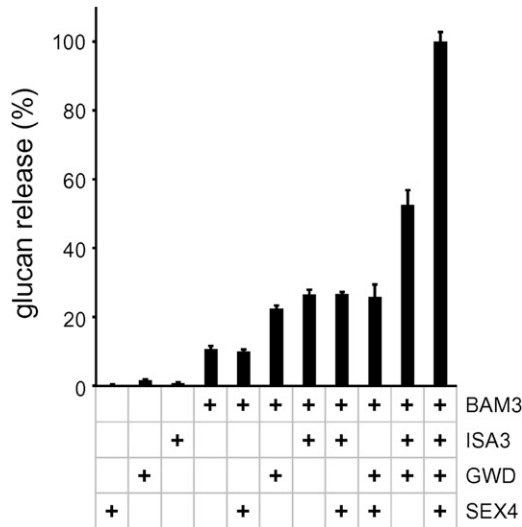


Figure 5. Hydrolytic Glucan Release Is Stimulated upon Simultaneous Phosphorylation and Dephosphorylation of Starch.

Phosphate-free starch granules from GWD-deficient *sex1-3* mutants were incubated with recombinant enzymes in the presence of 1 mM ATP. Glucan release into the supernatant was determined after acid hydrolysis. Since the absolute values varied significantly between individual experiments, glucan release was normalized to the maximum glucan release. Values are means \pm SE; n = 3 independent experiments, each with triplicate measurements.

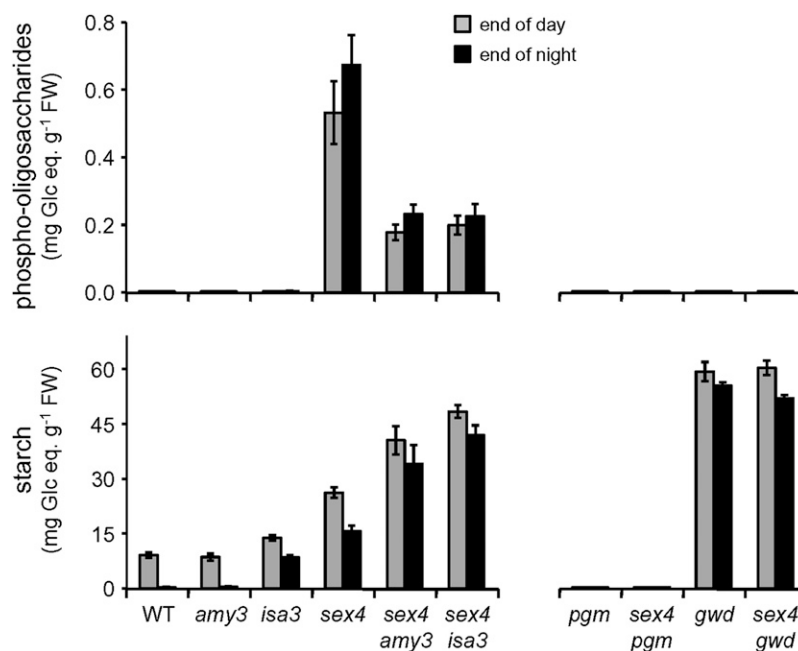


Figure 6. Mutation of *AMY3*, *ISA3*, *PGM*, or *GWD* Alters the Phenotype of *sex4* Plants.

Phospho-oligosaccharide and starch contents of 4-week-old plants (wild type and indicated mutants) were determined at the end of the day (gray bars) and the end of the night (black bars). Values are means \pm SE; $n = 5$ individual plants.

Third, analysis during the diurnal cycle shows that the phospho-oligosaccharide content of *sex4* leaves increases during starch breakdown at night and decreases during the day (Figure 3A). Fourth, the phospho-oligosaccharides accumulate in *sex4* plants as the leaves age (Figure 3B), correlating with the development of the starch-excess phenotype (Zeeman and ap Rees, 1999).

Given the reduced rate of starch breakdown in *sex4* leaves, we propose that the failure to remove the phosphates from the granule surface and/or from phospho-oligosaccharides inhibits the actions of starch-degrading enzymes. For example, exoacting β -amylases cannot pass phosphate residues and stop one or two residues before a phosphate group (Takeda and Hizukuri, 1981). Indeed, our results show that β -amylase cannot release detectable amounts of maltose from phospho-oligosaccharides in *sex4* extracts (see Supplemental Figure 2 online), suggesting that at least one phosphate group is esterified to a glucosyl residue near the nonreducing end of the chains. However, after dephosphorylation of the phospho-oligosaccharides in vitro, β -amylase was able to completely convert the resulting glucans to maltose and maltotriose (see Supplemental Figure 2 online), showing that they were linear (β -amylase cannot hydrolyze or act close to α -1,6 branch points).

The phospho-oligosaccharides in *sex4* are released from the granule surface at night via debranching (i.e., by *ISA3*) and endoamylolysis (i.e., by *AMY3*) (Figure 6). This is indicated by the reduction in the amount of phospho-oligosaccharides when either *ISA3* or *AMY3* are absent in addition to *SEX4*. Moreover, the observation that higher amounts of long phospho-oligosac-

charides (DP 15 to 25) are present in *sex4 amy3* than in *sex4* plants (Figure 7) indicates that these long chains can serve as substrates for *AMY3* in the *sex4* single mutant. In addition to releasing phospho-oligosaccharides from the granule surface, *AMY3* may therefore also have a function in the shortening of longer linear phospho-oligosaccharides in the stroma.

During the day, the phospho-oligosaccharide content of *sex4* leaves decreases by 30% (Figure 3A). This is unlikely to be due to the action of glucan degrading enzymes, as there is no shift of the chain length distribution toward shorter chains. Some phospho-oligosaccharides could be incorporated into the granule during starch biosynthesis in the light. For example, malto-oligosaccharides have been implicated in the priming of amylose synthesis (Denyer et al., 1996, 1999; Zeeman et al., 2002a), and it is worth noting that *sex4* starch has a high amylose content. Alternatively, other glucan phosphatases could dephosphorylate phospho-oligosaccharides to some extent, thereby making them accessible for exo-amylolytic degradation. The *Arabidopsis* genome contains two other genes encoding proteins with a phosphatase domain similar to *SEX4* (At3g01510 and At3g10940, having 33 and 45% amino acid identity with the *SEX4* phosphatase domain, respectively). The protein encoded by At3g01510 is a particularly good candidate for a second glucan phosphatase. In addition to the phosphatase domain, it has a predicted chloroplast transit peptide and a carbohydrate binding module similar to that of *SEX4* (35% amino acid identity). Mutation of this gene also results in elevated amounts of leaf starch, which is additive to the phenotype caused by loss of *SEX4* (S. Comparot-Moss, O. Kötting, S.C. Zeeman, and A.M. Smith, unpublished

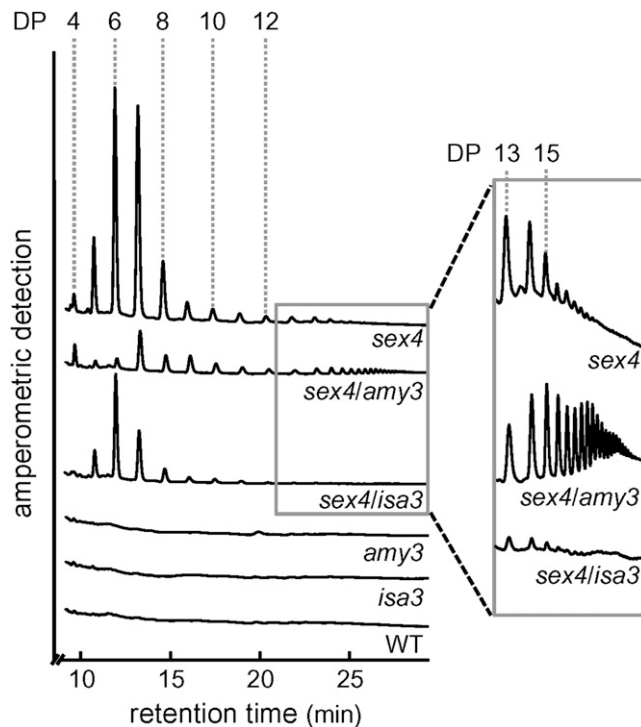


Figure 7. Phospho-Oligosaccharide Pattern in *sex4* Plants Is Altered upon Loss of *AMY3* or *ISA3* Expression.

Representative HPAEC-PAD chromatograms ($n = 5$ individual plants) of phosphatase-treated extracts from wild-type, *sex4*, *amy3*, *isa3*, and the respective double mutant plants harvested at the end of the night. Numbers above the panel indicate the DP of the detected (phospho-) glucans. The panel on the right represents a zoom into the region of late-eluting glucans.

data). The third putative phosphatase, At3g10940, does not possess a predicted chloroplast transit peptide or a carbohydrate binding module, and mutation of this gene does not result in altered starch levels (D. Santelia and S.C. Zeeman, unpublished data). Since there are two dikinases in *Arabidopsis* that exhibit specificity for the C6 or the C3 positions of glucosyl units in amylopectin (Ritte et al., 2006), it is tempting to speculate that the two plastidial phosphatases also have different substrate specificities. SEX4 might preferentially release phosphate esterified to the C6 position, whereas C3-bound phosphate could be preferentially released by the other related phosphatase. However, additional research will be required to evaluate this hypothesis.

Our work demonstrates unambiguously the phosphoglucan phosphatase activity of SEX4 (Figure 1) but does not definitively discriminate between the starch granule surface and phospho-oligosaccharides as the actual substrate of SEX4 in vivo. Although phospho-oligosaccharides accumulate in the absence of SEX4 (Figures 2 and 3), this does not necessarily mean that they are the actual substrate, as they might be released from the granule surface in significant amounts only when the surface itself is not efficiently dephosphorylated. Our in vitro studies with

recombinant enzymes provide support for the idea that the granule is in fact the true substrate. First, recombinant SEX4 promotes the degradation of starch granules by ISA3 and BAM3 when incubated together with GWD (and ATP) but not in the absence of GWD. The simplest explanation for this enhancing effect is that SEX4 dephosphorylates the granule surface and thereby allows it to be further hydrolyzed by BAM3 and ISA3. If SEX4 were only dephosphorylating phospho-oligosaccharides that had already been released into solution, no increase in released glucan would have been measured in our experiments. However, it is also possible that released phosphoglucans act as specific inhibitors of the hydrolases BAM3 and ISA3. Second, there is evidence for synergistic action between the enzymes of starch degradation and enzymes that phosphorylate glucans. Edner et al. (2007) showed that granule phosphorylation by GWD stimulates the activity of BAM1 and BAM3 on the starch granule and that pretreatment of the granules with BAM enhances the activity of GWD. Similarly, pretreatment of phosphorylated granules with BAM enhances the phosphatase activity of SEX4. This mutual enhancement suggests that cooperative activity may be central to the solubilisation and degradation of semicrystalline amylopectin.

Our model for the possible interplay between SEX4, GWD, β -amylase, and isoamylase is depicted in Figure 8 and builds upon previously published concepts (Edner et al., 2007; Zeeman et al., 2007). Phosphorylation by GWD and PWD could result in uncoiling of the double helices, allowing BAM to degrade the exposed chains (Figure 8). Indeed, a recent study provides direct evidence that PWD-catalyzed C3 phosphorylation disturbs the structure of amylopectin double helices (Hansen et al., 2008). However, since BAM can pass neither α -1,6 branches nor phosphate groups, maltose release will be limited (Takeda and Hizukuri, 1981; Edner et al., 2007). Removal of the phosphate group by SEX4 or branch points by ISA3 would enable BAM to further degrade the glucan chains. As a result of the starch degradation by BAM and ISA3, more glucan chains will be accessible for phosphorylation by GWD and PWD. It is plausible that such a cycle of phosphorylation, degradation, and dephosphorylation could occur during the degradation of the successive semicrystalline lamellae formed by the clusters of amylopectin. Our view of the situation in *sex4* mutants is also depicted in the model (Figure 8). Loss of SEX4 reduces the release of orthophosphate from the granule surface, thus hindering maltose release by β -amylase. Indeed, nighttime maltose levels are lower in *sex4* than in wild-type plants (Messerli et al., 2007). ISA3 and AMY3 now release both nonphosphorylated and phospho-oligosaccharides, and the latter accumulate in the chloroplast stroma. We emphasize that, in addition to SEX4, other phosphatases may be involved and we cannot exclude the possibility that SEX4 also acts on soluble phospho-oligosaccharides.

Other organisms have proteins with phosphatase domains with sequence similarity to SEX4 (i.e., laforin in vertebrates) (Gentry et al., 2007) and the capacity for glucan dephosphorylation in vitro (Worby et al., 2006). Recent reports have suggested that laforin is involved in glycogen dephosphorylation in mice (Tagliabracci et al., 2007) and, based on the apparent ability of the human laforin to rescue the high starch phenotype of *sex4*, that laforin and SEX4 may be functionally equivalent (Gentry

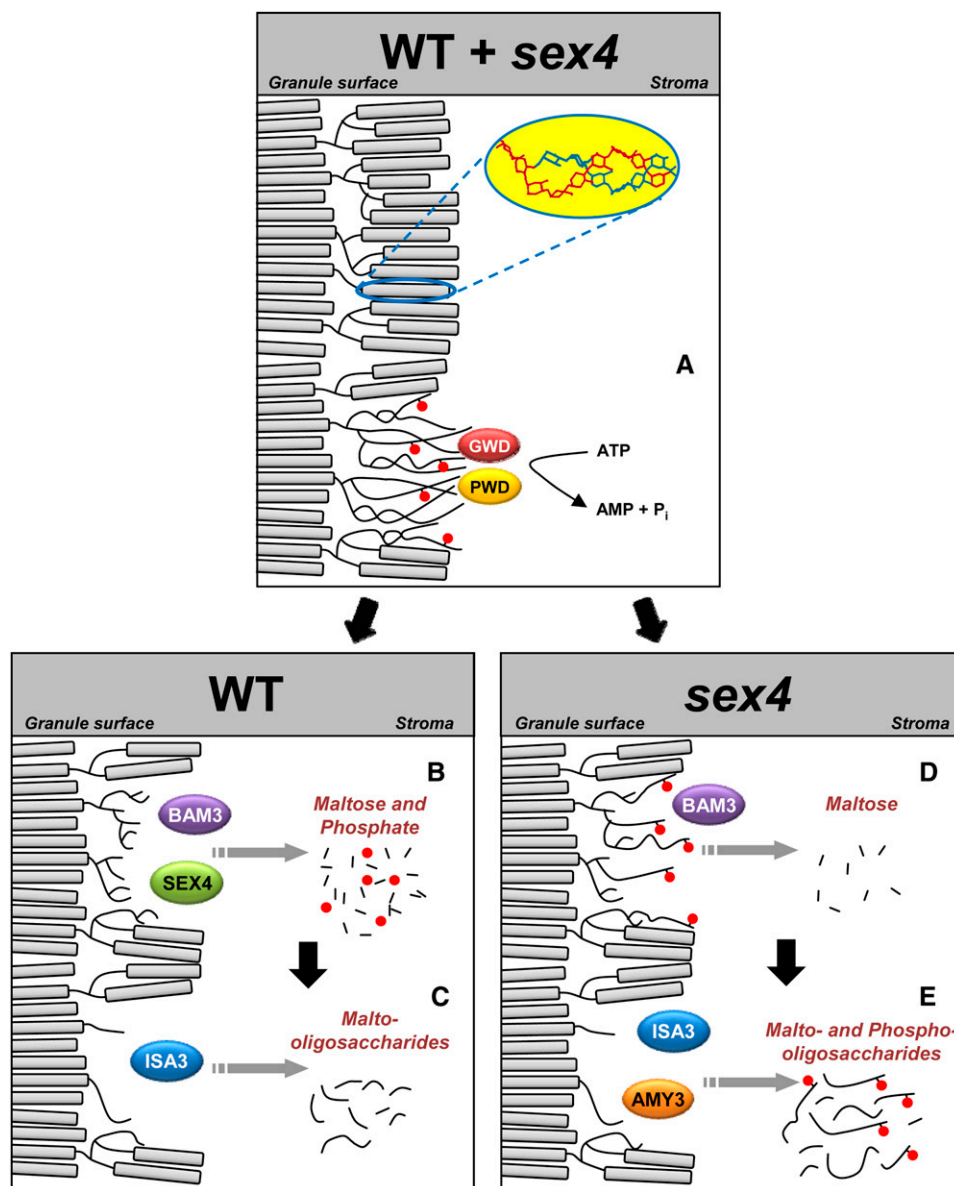


Figure 8. Proposed Model of the Initial Events at the Granule Surface during Starch Breakdown.

At night, starch is phosphorylated at the granule surface by GWD and PWD in both wild-type and *sex4* plants (A), leading to partial unwinding of amylopectin double helices. In the wild type, BAM3 and SEX4 can subsequently release maltose and phosphate, respectively (B), while ISA3 hydrolyzes branch points and releases malto-oligosaccharides (C). In *sex4* mutants, phosphate is not removed by SEX4, leading to reduced maltose release by BAM3 (D). The following actions of ISA3 and AMY3 release malto- and phospho-oligosaccharides. After degradation of one semicrystalline lamella, a new cycle can start again with the phosphorylation of the granule surface by GWD and PWD (A).

et al., 2007). Unlike amylopectin, glycogen is generally regarded as soluble. Thus, it is not clear whether glucan phosphorylation would be required to disrupt secondary structures as is proposed for starch. However, it is possible that phosphorylation may help to resolubilize insoluble structures that form and that cannot be remobilized by the glycogen degrading enzymes. Laforin would thus play an analogous role to that of SEX4 in

amylopectin metabolism. This hypothesis is supported by the fact that highly phosphorylated, insoluble LBs accumulate in vertebrates if the glucan phosphatase laforin is missing and the recent proposition that laforin functions to suppress excessive glycogen phosphorylation (Tagliabracci et al., 2008). As yet, it is unclear which protein is responsible for glycogen phosphorylation in animals, as GWD and PWD are plant specific.

METHODS

Plants and Growth Conditions

Plants for metabolite measurements were grown in a controlled environment chamber (AR-95L; Percival Scientific) in a 12-h/12-h light/dark regime with a constant temperature of 20°C, 60% relative humidity, and a uniform illumination of 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Plants used for the preparation of leaf starch granules were grown in a greenhouse (20°C/16°C day/night) with additional 12-h illumination during the day. *Arabidopsis thaliana* (ecotype Columbia) was used as the wild-type reference. All mutants used here were created in this background. The following *Arabidopsis* T-DNA insertion mutants were used in this study: *sex4-3* (Salk_102567) (Niittylä et al., 2006), *amy3-2* (SAIL_613 D12) (Yu et al., 2005), *isa3-2* (GABI_280G10) (Delatte et al., 2006), and *sex1-8* (= *gwd*; Salk_077211) (Ritte et al., 2006). The *pgm-1* (Caspar et al., 1985; Periappuram et al., 2000), *sex4-2* (Niittylä et al., 2006), and *sex1-3* (Yu et al., 2001) mutants were previously identified in phenotypic screens of populations of ethyl methane sulfonate and x-ray-mutagenized *Arabidopsis* seeds.

Production and Selection of Multiple-Mutant Lines

Double mutants were obtained by crossing and selecting homozygous plants of the required genotypes from the segregating F2 populations using PCR- and cleaved-amplified polymorphic sequence-based genotyping. To control for the presence of unlinked secondary mutations that might affect the mutant phenotypes, we routinely confirmed that the offspring of different isolates of each genotype were phenotypically similar in terms of growth rate, starch content, and phospho-oligosaccharide content. The insertion sites of the T-DNAs from the Salk collection were characterized using the T-DNA-specific primer 5'-GCGTGAC-CGCTTGCTGCAACT-3' in combination with the appropriate gene-specific primers as follows: *SEX4*-specific primers, 5'-AAGCTGATGCG-TAATGAATCG-3' and 5'-CACTAGTAAAACATGATTAACATGTTGG-3'; and *GWD*-specific primers, 5'-TCCGGTATGACAAGTCGAATC-3' and 5'-GTCAGTCTATCTCGCGCTTTC-3'. The insertion site of the T-DNA from the Syngenta SAIL collection (Torrey Mesa Research Institute, San Diego, CA) was characterized using the T-DNA-specific primer 5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3' in combination with the appropriate gene-specific primers as follows: *AMY3*-specific primers, 5'-CCGACCTTGTAATTTCTTCACTG-3' and 5'-GGTTCCTC-TTGTAGACGATGTTCC-3'. The insertion site of the T-DNA from the GABI_Kat collection (Rosso et al., 2003) was characterized using the T-DNA-specific primer 5'-ATATTGACCATCATCTCATTGC-3' in combination with the appropriate gene-specific primers as follows: *ISA3*-specific primers, 5'-ACTGGAGAAGGATTGAAGAGAATG-3' and 5'-GATCTTCCGATTTTACCAGGTTA-3'. The mutation in *sex4-2* was identified by amplifying a 239-bp fragment with the *SEX4*-specific primers 5'-TGAGACTGTTACGAGCACATGC-3' and 5'-GTAACATACCGCAA-CAGCAGGGGCC-3'. The wild-type amplicon is susceptible to digestion with *Apal*, whereas the *sex4-2* fragment is not. The mutation in *pgm-1* was identified by amplifying a 1538-bp fragment with the *PGM*-specific primers 5'-AGGCTTCCGAGCAACTCAATATC-3' and 5'-CTGACCAC-TGCTGTAATTGAAC-3'. The *pgm-1* amplicon is susceptible to digestion with *BspCNI*, whereas the wild-type fragment is not. The *sex4 pgm* double mutant was created by crossing *sex4-2* and *pgm-1* plants, and all other double mutants were created using the *sex4-3* line. The *sex4* single mutant analyses of starch and phospho-oligosaccharide contents were done using both the *sex4-3* and *sex4-2* lines and yielded identical results; however, only *sex4-3* analyses are shown here.

Recombinant Proteins

The *Arabidopsis* *SEX4* protein and the active-site mutant version (*SEX4 C/S*), both lacking the first 52 amino acids (the first 54 amino acids

comprise the chloroplast transit peptide), were produced as described previously (Gentry et al., 2007). Production of recombinant potato (*Solanum tuberosum*) *GWD* and *ISA3* and *Arabidopsis* *BAM3* was as described previously (Ritte et al., 2000, 2002; Hussain et al., 2003; Edner et al., 2007).

Preparation of Leaf Starch Granules

Whole rosettes of 4- to 5-week-old plants from the greenhouse were harvested after 10 h of illumination. Harvested plants were immediately frozen in liquid nitrogen and stored at -80°C. The subsequent preparation of leaf starch granules was performed as described previously (Kötting et al., 2005).

Extraction and Measurement of Starch and Phospho-Oligosaccharides

For starch and phospho-oligosaccharide analyses, whole rosettes of 4- to 5-week-old plants were harvested at the times indicated and immediately frozen in liquid nitrogen. Samples were homogenized using an all-glass homogenizer in 1 M perchloric acid. Soluble and insoluble fractions were separated by centrifugation (3000g, 15 min, 4°C). Insoluble material, including starch, was resuspended once in water to remove residual soluble glucans, at least three times in 80% (v/v) ethanol (20°C), and finally resuspended in water and stored at -20°C. Starch measurement was performed as described (Smith and Zeeman, 2006). The soluble fraction, including phospho-oligosaccharides, was adjusted to pH 5.0 by adding 2 M KOH, 0.4 M MES, and 0.4 M KCl. Precipitated potassium perchlorate was removed by centrifugation (20,000g, 15 min, 4°C), and extracts were stored at -20°C.

Phospho-oligosaccharide samples for HPAEC-PAD analysis were treated as follows: first, an aliquot of the neutralized soluble fraction corresponding to 3 mg fresh weight was incubated in 0.1 mL 1× Antarctic Phosphatase buffer (New England Biolabs) with or without (mock) 15 units of Antarctic Phosphatase (New England Biolabs) for 2 h at 37°C. Glucose 6-phosphate standards treated with Antarctic Phosphatase under the same conditions were 95% dephosphorylated as revealed by enzymatic determination of glucose and glucose-6-phosphate of the products as described (Smith and Zeeman, 2006). Subsequently, the samples were purified by ion exchange on sequential columns of Dowex 50W and Dowex 1 (Sigma-Aldrich) with a 2-mL bed volume each as follows: sample volume was made to 0.5 mL with water, applied to the column, the neutral compounds were eluted with 5 mL water, lyophilized, and redissolved in 0.1 mL of water. Afterwards, phosphatase- and mock-treated samples were separated by HPAEC-PAD on a CarboPac PA20 column (Dionex) according to the following conditions: injection volume, 10 μL ; flow rate, 0.5 mL min⁻¹; eluent A, 100 mM NaOH; eluent B, 100 mM NaOH and 50 mM sodium acetate; eluent C, 150 mM NaOH and 500 mM sodium acetate. The gradient was as follows: 0 to 3 min, 50% A and 50% B; 3 to 20 min, a concave gradient to 50% A, 10% B, and 40% C (malto-oligosaccharide elution); 20 to 29.5 min, convex gradient to 10% B and 90% C (column wash step); 36 to 36.5 min, step to 50% A and 50% B; 36.5 to 40 min, 50% A and 50% B (column reequilibration). Peaks with a DP of 1 to 7 were identified by comparison with external malto-oligosaccharide standards and quantified with Chromeleon software (Dionex). The peaks with DP 8 to 20 were quantified by extrapolation of the DP 1 to 7 standards and therefore represent an estimation. The phospho-oligosaccharide content given is the sum of the chains with DP 4 to 20.

Determination of Starch-Bound Phosphate

For the analysis of starch-bound phosphate, 5 mg of starch granules were solubilized in 100 μL 1 M HCl for 2 h at 95°C. After neutralization with 100

μL 1 M NaOH, 50 μL solubilized starch were incubated with 15 units of Antarctic Phosphatase (New England Biolabs) in 1 \times Antarctic Phosphatase reaction buffer from the supplier for 2 h at 37°C. Orthophosphate was determined with malachite green as described (Werner et al., 2005). Since only 95% of the phosphate esters are hydrolyzed under the conditions used (see above), we corrected the determined orthophosphate values to 100%.

Release of ^{33}P from Prelabeled Starch Granules

Purified starch granules from the GWD-deficient *Arabidopsis* mutants *sex1-3* (Yu et al., 2001) or *gwd* (= *sex1-8*) (Ritte et al., 2006) were phosphorylated in vitro with recombinant potato GWD (Ritte et al., 2002) and [β - ^{33}P]ATP as phosphate donor as described (Edner et al., 2007). Phosphate incorporation into starch by GWD was 17.3 $\mu\text{mol P mol}^{-1}$ Glc equivalents (i.e., 1 phosphate per 58,000 glucosyl residues), which is much lower than the level of phosphate in wild-type *Arabidopsis* starch (~ 1 in 2000 glucosyl residues) (Yu et al., 2001).

For the preparation of crude extracts, whole rosettes of 4- to 5-week-old *Arabidopsis* wild-type and *sex4-3* mutant plants were harvested at the end of the light period, snap-frozen in liquid nitrogen, and stored at -80°C until extraction. Three grams of leaf material was extracted in 6 mL ice-cold extraction medium (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 2 mM DTT, 2 mM benzamidine, 2 mM ϵ -aminocaproic acid, and 0.5 mM phenylmethylsulfonylfluoride) with mortar and pestle. The homogenate was centrifuged (4°C , 15 min, 20,000g), and the supernatant was desalted with PD-10 columns (GE Healthcare) into extraction medium, snap-frozen in liquid nitrogen, and stored at -80°C until use. Release of ^{33}P from prelabeled granules by plant extracts was determined as follows: 0.5 mg of ^{33}P -labeled starch (equivalent to $\sim 0.15 \mu\text{Ci}$) was incubated with the desalted extracts (equivalent to 20 μg total protein) in reaction medium (50 mM HEPES-KOH, pH 7.5, 5 mM MgCl_2 , 5 mM CaCl_2 , 1 mg mL^{-1} BSA, and 2 mM DTT) in a final volume of 90 μL for 25 min at 25°C . Phosphate release over time was linear under these conditions. The reaction was stopped by the addition of 10% (w/v) aqueous SDS to a final concentration of 2.5%. After centrifugation (5 min, 16,000g), release of ^{33}P was determined by subjecting an aliquot of the supernatant to scintillation counting.

Release of ^{33}P from prelabeled granules by recombinant SEX4 protein was determined as follows: recombinant SEX4 or the active-site mutant protein SEX4 C/S (0.5 $\mu\text{g mL}^{-1}$ each) was incubated in dephosphorylation medium (0.1 M sodium acetate, 50 mM bis-Tris, 50 mM Tris-HCl, pH 6.0, 2 mM DTT, and 1 mg mL^{-1} BSA) containing different concentrations of prelabeled starch granules (2 to 8 mg mL^{-1}) in a volume of 0.9 mL at 24 to 26°C on a rotating wheel. Reactions were stopped after 1 to 20 min by mixing a 150- μL aliquot with 50 μL of 10% SDS. After centrifugation (5 min, 16,000g), release of ^{33}P was determined by subjecting an aliquot of the supernatant to scintillation counting.

Isolation, Enrichment, and MS Analysis of Phospho-Oligosaccharides

Whole rosettes of 4-week-old *sex4* plants were harvested at the end of the night, frozen in liquid nitrogen, and homogenized with a mortar and pestle. Extraction was performed twice in boiling 80% (v/v) ethanol ($2 \times 3 \text{ mL}$, 80°C , 15 min). The supernatants were combined, adjusted to 6 mL with ethanol, and mixed with 2 mL trichloromethane. Water was added (3.5 mL), whirled for 10 s, and centrifuged (3000g, 10 min). The upper water phase was transferred to a new tube and dried in a vacuum concentrator. Dried material was redissolved in 1 mL of water. Enrichment of phospho-oligosaccharides on prepacked nonporous graphitized carbon black columns (Carbograp SPE; Alltech) was essentially as described (Hejazi et al., 2008); however, sample volume was 0.9 mL. MS

analysis of the enriched phospho-oligosaccharides was as described (Hejazi et al., 2008).

Glucan Release from Starch Granules

Phosphate-free starch granules from the *Arabidopsis* *sex1-3* mutant (2.5 mg) were incubated with different recombinant enzymes (60 min, 25°C , shaking) in reaction medium (50 mM HEPES-KOH, pH 7.5, 5 mM CaCl_2 , 5 mM MgCl_2 , 1 mg mL^{-1} BSA, 1 mM ATP, and 2 mM DTT; final volume: 120 μL). Recombinant enzymes were used at the following concentrations: ISA3, 8.3 $\mu\text{g mL}^{-1}$; GWD, BAM3, and SEX4, 16.6 $\mu\text{g mL}^{-1}$. Incubation was stopped by heating (5 min, 95°C) of the supernatant after centrifugation (1 min, 16,000g). Following acid hydrolysis (final concentration: 1 M HCl, 2 h, 100°C) and neutralization of the supernatant, glucan release was determined as glucose, as described (Kötting et al., 2005).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *SEX4*, At3g52180; *GWD*, At1g10760; *BAM3*, At4g17090; *ISA3*, At4g09020; *AMY3*, At1g69830; and *PGM*, At5g51820.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Recombinant SEX4 Protein Can Act on Phospho-Oligosaccharides.

Supplemental Figure 2. Phospho-Oligosaccharides in *sex4* Are Resistant to β -Amylase Treatment.

ACKNOWLEDGMENTS

We thank Jing Li and Steven M. Smith (University of Western Australia, Crawley, Australia) for the BAM3 expression vector, Cathie Martin and Hasnain Hussain (John Innes Centre, Norwich, UK) for the potato ISA3 expression vector, Tien-Shin Yu (Academia Sinica, Taipei, Taiwan) for the *sex4 pgm* double mutant, and Sebastian Streb (ETH Zurich, Zurich, Switzerland) for technical assistance with HPAEC-PAD analyses. This work was funded by the ETH Zurich, the Roche Research Foundation, and the National Centre for Competence in Research-Plant Survival (Swiss National Science Foundation). The Deutsche Forschungsgemeinschaft supported this work within the SFB 429 TP B2 (M.S.) and B7 (C.E. and G.R.). M.S.G. is supported by National Institute of Health Grant 5R00NS061803 and the University of Kentucky. S.C.-M. and A.M. S. are supported by Grants BBS/B/11877 and BB/C513542/1, respectively, awarded to the John Innes Centre by the Biotechnology and Biological Sciences Research Council (UK). J.C. is supported by the National Science Council, Taiwan (Grant NSC-96-2311-B-001-004).

Received November 17, 2008; revised December 18, 2008; accepted December 23, 2008; published January 13, 2009.

REFERENCES

- Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004). Protein tyrosine phosphatases in the human genome. *Cell* **117**: 699–711.

- Baunsgaard, L., Lütken, H., Mikkelsen, R., Glaring, M.A., Pham, T.T., and Blennow, A. (2005). A novel isoform of glucan, water dikinase phosphorylates pre-phosphorylated α -glucans and is involved in starch degradation in *Arabidopsis*. *Plant J.* **41**: 595–605.
- Blennow, A., Nielsen, T.H., Baunsgaard, L., Mikkelsen, R., and Engelsen, S.B. (2002). Starch phosphorylation: A new front line in starch research. *Trends Plant Sci.* **7**: 445–450.
- Caspar, T., Huber, S.C., and Somerville, C. (1985). Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. *Plant Physiol.* **79**: 11–17.
- Delatte, T., Umhang, M., Trevisan, M., Eicke, S., Thorneycroft, D., Smith, S.M., and Zeeman, S.C. (2006). Evidence for distinct mechanisms of starch granule breakdown in plants. *J. Biol. Chem.* **281**: 12050–12059.
- Denyer, K., Clarke, B., Hylton, C., Tatge, H., and Smith, A.M. (1996). The elongation of amylose and amylopectin chains in isolated starch granules. *Plant J.* **10**: 1135–1143.
- Denyer, K., Waite, D., Edwards, A., Martin, C., and Smith, A.M. (1999). Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. *Biochem. J.* **342**: 647–653.
- Edner, C., Li, J., Albrecht, T., Mahlow, S., Hejazi, M., Hussain, H., Kaplan, F., Guy, C., Smith, S.M., Steup, M., and Ritte, G. (2007). Glucan, water dikinase activity stimulates breakdown of starch granules by plastidial beta-amylases. *Plant Physiol.* **145**: 17–28.
- Fordham-Skelton, A.P., Chilley, P., Lumberras, V., Reignoux, S., Fenton, T.R., Dahm, C.C., Pages, M., and Gatehouse, J.A. (2002). A novel higher plant protein tyrosine phosphatase interacts with SNF1-related protein kinases via a KIS (kinase interaction sequence) domain. *Plant J.* **29**: 705–715.
- Gentry, M.S., Down, R.H., Worby, C.A., Mattoo, S., Ecker, J.R., and Dixon, J.E. (2007). The phosphatase laforin crosses evolutionary boundaries and links carbohydrate metabolism to neuronal disease. *J. Cell Biol.* **178**: 477–488.
- Hansen, P.I., Spraul, M., Dvorsak, P., Larsen, F.H., Blennow, A., Motawia, M.S., and Engelsen, S.B. (2008). Starch phosphorylation - Maltosidic restrains upon 3'- and 6'-phosphorylation investigated by chemical synthesis, molecular dynamics and NMR spectroscopy. *Biopolymers* **91**: 179–193.
- Harriman, D.G., Millar, J.H., and Stevenson, A.C. (1955). Progressive familial myoclonic epilepsy in three families: Its clinical features and pathological basis. *Brain* **78**: 325–349.
- Hejazi, M., Fettke, J., Haebel, S., Edner, C., Paris, O., Froberg, C., Steup, M., and Ritte, G. (2008). Glucan, water dikinase phosphorylates crystalline maltodextrins and thereby initiates solubilization. *Plant J.* **55**: 323–334.
- Hussain, H., Mant, A., Seale, R., Zeeman, S.C., Hinchliffe, E., Edwards, A., Hylton, C., Bornemann, S., Smith, A.M., Martin, C., and Bustos, R. (2003). Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. *Plant Cell* **15**: 133–149.
- Kerk, D., Conley, T.R., Rodriguez, F.A., Tran, H.T., Nimick, M., Muench, D.G., and Moorhead, G.B.G. (2006). A chloroplast-localized dual-specificity protein phosphatase in *Arabidopsis* contains a phylogenetically dispersed and ancient carbohydrate-binding domain, which binds the polysaccharide starch. *Plant J.* **46**: 400–413.
- Kötting, O., Pusch, K., Tiessen, A., Geigenberger, P., Steup, M., and Ritte, G. (2005). Identification of a novel enzyme required for starch metabolism in *Arabidopsis* leaves. The phosphoglucan, water dikinase. *Plant Physiol.* **137**: 242–252.
- Lafora, G.R., and Glück, B. (1911). Beitrag zur Histopathologie der myoklonischen Epilepsie. *Z. Ges. Neurol. Psychiatr.* **6**: 1–14.
- Liu, R., Wang, L., Chen, C., Liu, Y., Zhou, P., Wang, Y., Wang, X., Turnbull, J., Minassian, B.A., and Zheng, P. (2008). Laforin negatively regulates cell cycle progression through GSK-3 β dependent mechanisms. *Mol. Cell. Biol.* **28**: 7236–7244.
- Lloyd, J.R., Kossmann, J., and Ritte, G. (2005). Leaf starch degradation comes out of the shadows. *Trends Plant Sci.* **10**: 130–137.
- Lorberth, R., Ritte, G., Willmitzer, L., and Kossmann, J. (1998). Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. *Nat. Biotechnol.* **16**: 473–477.
- Luan, S. (2003). Protein phosphatases in plants. *Annu. Rev. Plant Biol.* **54**: 63–92.
- Messerli, G., Partovi Nia, V., Trevisan, M., Kolbe, A., Schauer, N., Geigenberger, P., Chen, J., Davison, A.C., Fernie, A.R., and Zeeman, S.C. (2007). Rapid classification of phenotypic mutants of *Arabidopsis* via metabolite fingerprinting. *Plant Physiol.* **143**: 1484–1492.
- Minassian, B.A., et al. (1998). Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. *Nat. Genet.* **20**: 171–174.
- Niittylä, T., Comparot-Moss, S., Lue, W.L., Messerli, G., Trevisan, M., Seymour, M.D., Gatehouse, J.A., Villadsen, D., Smith, S.M., Chen, J., Zeeman, S.C., and Smith, A.M. (2006). Similar protein phosphatases control starch metabolism in plants and glycogen metabolism in mammals. *J. Biol. Chem.* **281**: 11815–11818.
- Periappuram, C., Steinhauer, L., Barton, D.L., Taylor, D.C., Chatson, B., and Zou, J. (2000). The plastidic phosphoglucomutase from *Arabidopsis*. A reversible enzyme reaction with an important role in metabolic control. *Plant Physiol.* **122**: 1193–1199.
- Pulido, R., and Hooft van Huijsduijnen, R. (2008). Protein tyrosine phosphatases: Dual-specificity phosphatases in health and disease. *FEBS J.* **275**: 848–866.
- Ritte, G., Heydenreich, M., Mahlow, S., Haebel, S., Kötting, O., and Steup, M. (2006). Phosphorylation of C6- and C3-positions of glucosyl residues in starch is catalysed by distinct dikinases. *FEBS Lett.* **580**: 4872–4876.
- Ritte, G., Lloyd, J.R., Eckermann, N., Rottmann, A., Kossmann, J., and Steup, M. (2002). The starch-related R1 protein is an α -glucan, water dikinase. *Proc. Natl. Acad. Sci. USA* **99**: 7166–7171.
- Ritte, G., Lorberth, R., and Steup, M. (2000). Reversible binding of the starch-related R1 protein to the surface of transitory starch granules. *Plant J.* **21**: 387–391.
- Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol. Biol.* **53**: 247–259.
- Sakai, M., Austin, J., Witmer, F., and Trueb, L. (1970). Studies in myoclonus epilepsy (Lafora body form). II. Polyglucosans in the systemic deposits of myoclonus epilepsy and in corpora amylacea. *Neurology* **20**: 160–176.
- Schnabel, R., and Seitelberger, F. (1968). Histophysical and histochemical investigations of myoclonus bodies. *Pathol. Eur.* **3**: 218–226.
- Serratos, J.M., Delgado-Escueta, A.V., Posada, I., Shih, S., Drury, I., Berciano, J., Zabala, J.A., Antunez, M.C., and Sparkes, R.S. (1995). The gene for progressive myoclonus epilepsy of the Lafora type maps to chromosome 6q. *Hum. Mol. Genet.* **4**: 1657–1663.
- Serratos, J.M., et al. (1999). A novel protein tyrosine phosphatase gene is mutated in progressive myoclonus epilepsy of the Lafora type (EPM2). *Hum. Mol. Genet.* **8**: 345–352.
- Singh, S., Satishchandra, P., Shankar, S.K., and Ganesh, S. (2008). Lafora disease in the Indian population: EPM2A and NHLRC1 gene mutations and their impact on subcellular localization of laforin and malin. *Hum. Mutat.* **29**: E1–E12.

- Smith, A.M., and Zeeman, S.C.** (2006). Quantification of starch in plant tissues. *Nat. Protocols* **1**: 1342–1345.
- Smith, A.M., Zeeman, S.C., and Smith, S.M.** (2005). Starch degradation. *Annu. Rev. Plant Biol.* **56**: 73–97.
- Sokolov, L.N., Dominguez-Solis, J.R., Allary, A.L., Buchanan, B.B., and Luan, S.** (2006). A redox-regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation. *Proc. Natl. Acad. Sci. USA* **103**: 9732–9737.
- Solaz-Fuster, M.C., et al.** (2008). Regulation of glycogen synthesis by the laforin-malin complex is modulated by the AMP-activated protein kinase pathway. *Hum. Mol. Genet.* **17**: 667–678.
- Tagliabracci, V.S., Girard, J.M., Segvich, D., Meyer, C., Turnbull, J., Zhao, X., Minassian, B.A., Depaoli-Roach, A.A., and Roach, P.J.** (2008). Abnormal metabolism of glycogen phosphate as a cause for lafora disease. *J. Biol. Chem.* **283**: 33816–33825.
- Tagliabracci, V.S., Turnbull, J., Wang, W., Girard, J.M., Zhao, X., Skurat, A.V., Delgado-Escueta, A.V., Minassian, B.A., Depaoli-Roach, A.A., and Roach, P.J.** (2007). Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proc. Natl. Acad. Sci. USA* **104**: 19262–19266.
- Takeda, Y., and Hizukuri, S.** (1981). Studies on starch phosphate. Part 5. Reexamination of the action of sweet-potato beta-amylase on phosphorylated (1->4)- α -D-glucan. *Carbohydr. Res.* **89**: 174–178.
- Werner, T.P., Amrhein, N., and Freimoser, F.M.** (2005). Novel method for the quantification of inorganic polyphosphate (iPoP) in *Saccharomyces cerevisiae* shows dependence of iPoP content on the growth phase. *Arch. Microbiol.* **184**: 129–136.
- Worby, C.A., Gentry, M.S., and Dixon, J.E.** (2006). Laforin, a dual specificity phosphatase that dephosphorylates complex carbohydrates. *J. Biol. Chem.* **281**: 30412–30418.
- Yokoi, S., Austin, J., and Witmer, F.** (1967). Isolation and characterization of Lafora bodies in two cases of myoclonus epilepsy. *J. Neuropathol. Exp. Neurol.* **26**: 125–127.
- Yokoi, S., Austin, J., Witmer, F., and Sakai, M.** (1968). Studies in myoclonus epilepsy (Lafora body form). I. Isolation and preliminary characterization of Lafora bodies in two cases. *Arch. Neurol.* **19**: 15–33.
- Yu, T.S., et al.** (2001). The *Arabidopsis* *sex1* mutant is defective in the R1 protein, a general regulator of starch degradation in plants, and not in the chloroplast hexose transporter. *Plant Cell* **13**: 1907–1918.
- Yu, T.S., et al.** (2005). α -Amylase is not required for breakdown of transitory starch in *Arabidopsis* leaves. *J. Biol. Chem.* **280**: 9773–9779.
- Zeeman, S.C., and ap Rees, T.** (1999). Changes in carbohydrate metabolism and assimilate export in starch-excess mutants of *Arabidopsis*. *Plant Cell Environ.* **22**: 1445–1453.
- Zeeman, S.C., Delatte, T., Messerli, G., Umhang, M., Stettler, M., Mettler, T., Streb, S., Reinhold, H., and Kötting, O.** (2007). Starch breakdown: Recent discoveries suggest distinct pathways and novel mechanisms. *Funct. Plant Biol.* **34**: 465–473.
- Zeeman, S.C., Northrop, F., Smith, A.M., and ap Rees, T.** (1998). A starch-accumulating mutant of *Arabidopsis thaliana* deficient in a chloroplastic starch-hydrolysing enzyme. *Plant J.* **15**: 357–365.
- Zeeman, S.C., Smith, S.M., and Smith, A.M.** (2002a). The priming of amylose synthesis in *Arabidopsis* leaves. *Plant Physiol.* **128**: 1069–1076.
- Zeeman, S.C., Smith, S.M., and Smith, A.M.** (2004). The breakdown of starch in leaves. *New Phytol.* **163**: 247–261.
- Zeeman, S.C., Tiessen, A., Pilling, E., Kato, K.L., Donald, A.M., and Smith, A.M.** (2002b). Starch synthesis in *Arabidopsis*. Granule synthesis, composition, and structure. *Plant Physiol.* **129**: 516–529.